Standard Operating Procedure for Chlorophyll-*a* and Pheophytin-*a* (Turner Designs Method)

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1.0 Scope and Application

This method is applicable to waters from the Great Lakes and Tributary streams. The description is for 90% buffered acetone and fine mesh glass fiber filters.

2.0 Summary of Method

A representative sample of algae is collected on a filter by vacuum filtration in dim light. The filter is then placed in a screw cap culture tube in the dark. The tube is stored, in the dark, at sub-freezing temperatures until the time of analysis. At the time of analysis, 10 mL of 90% buffered acetone is added to the tube. Each acetone filled tube is placed in an ultrasonic bath, filled with ice and sonicated, for 20 minutes after which the tube is steeped, at 0°C, between 16 to 24 hours. The tube is centrifuged prior to determination of the fluorescence. Prescribed optical filters are used to determine the excitation and emission wavelengths (approx. 420 nm and 670 nm). The assay of chlorophyll-*a* is calculated from the decrease in fluorescence caused by acidification. The pheophytin-*a* is calculated from the residual fluorescence after accounting for that produced by the chlorophyll-*a*.

3.0 Sample Handling and Preservation

3.1 The entire procedure should be carried out as much as possible in subdued light (Green) to prevent photodecomposition. The frozen samples should also be protected from light during storage for the same reason.

3.2 To prevent a chlorophyll-*a* degredation product, pheophytin, all glassware should be clean and acid-free.

4.0 Interferences

Though chlorophyll-*b*, -*c*, pheophytin-*b*, -*c* and other organic materials interfere they are assumed to be at concentrations not considered significant.

5.0 Equipment Required

- Turner Designs model 10-AU filter fluorometer with appropriate filters
- Plastic filter funnel, Gelman
- Vacuum system (3-4 psi)
- GF/F filters, Whatman (47 mm)
- 16 X 100 mm screw cap culture tubes
- 13 X 100 mm culture tubes, disposable
- 250 mL filter flask with sidearm
- Nalgene Tubing

- (1) 200 mL volumetric flask
- (5) 100 mL volumetric flask
- Aluminum Foil
- Parafilm
- Spectrophotometer
- Disposable glass pipets
- Chlorophyll-*a* standard (substantially free of chlorophyll *b*)
- High purity grade acetone (1 L)
- Magnesium Carbonate
- Concentrated HCL
- Plotter
- Filter forceps
- Vacuum pressure

6.0 Reagents

6.1 *Saturated Magnesium Carbonate Solution*: Add 10 gram magnesium carbonate to 1000 mL of deionized water. The solution is allowed to settle for a minimum of 24 hours. *Only the clear "powder free" solution is used during subsequent steps***.**

6.2 *90% (v/v) Buffered Acetone*: Add 100 mL of the Magnesium Carbonate solution (Section 6.1) to 900 mL of Acetone in 1 L volumetric flask.

6.3 *0.1 N Hydrochloric Acid solution:* Add 8.5 mL of concentrated hydrochloric acid to 800 mL of deionized water in 1 liter volumetric flask. Adjust volume to 1 L with deionized water.

7.0 Calibration and QC Check Standards

7.1 *Chlorophyll-a Calibration Stock Standard*: In subdued light, before breaking the tip of the ampule. Weigh the ampule and its contents to the nearest 0.1 mg. Carefully break the tip of the ampule. Transfer the entire contents of the ampule into a 200 mL volumetric flask. Carefully rinse the ampule and inside of tip with 90% Buffered Acetone (Section 6.2), at least three times, into the 200 mL volumetric flask. Adjust volume to 200 mL in flask with buffered acetone (Section 6.2).

Store the broken ampule and tip until all of the residual acetone has evaporated. Reweigh the empty ampule and tip. Determine by difference the weight of chlorophyll-*a* added to the flask.

7.1.1 Determine the purity of the chlorophyll solution spectrophotometrically. Measure the Optical Density (O.D.) of the Stock solution (Section 7.1) on the spectrophotometer at 663 nm. Use the equation below to calculate chlorophyll-*a* concentration.

Chlorophyll-*a* (mg/L) = 11.42 X $O.D._{663}$

11.42 = extinction coefficient of chlorophyll-*a* at 663 nm

Note: If the concentration is 5 mg/L (1 mg/200 mL) and the purity is 100% then the O.D.

should be 0.4378.

7.2 Chlorophyll-*a* Intermediate Calibration Standard (2000 µg/L)

Dilute the Chlorophyll-*a* Stock Standard to a concentration of 2000 µg/L. Prepare at least 50 mL of solution.

7.3 Chlorophyll-*a* Working Calibration Standards:

mL of Intermediate Solution Std. (Section 7.2.1) diluted to 100 mL	Concentration of Calibration Std. µg/L Chlorophyll-*a*
4.0	80
0.0	0

7.4 Chlorophyll-*a* Working QC Check Standards:

mL of Intermediate Solution Std. (Section 7.2) diluted to 100 mL	Concentration of QC Check Std. µg/L Chlorophyll-*a*
5.0	100
0.5	10

8.0 Calibration and Standardization

8.1 Calibration should be done each time a batch of samples are analyzed. Allow the instrument to warm-up for at least 15 minutes. See the Turner Design's Model 10-AU-005 Field Fluorometer User's Manual Section 3 (Method B) for a full discussion of instrument calibration instructions.

Samples and standards are to be maintained at the same temperature by using a cooler filled with ice.

8.2 To measure pheophytin-*a*, it will be necessary to obtain before-to after acidification response ratios of the chlorophyll-*a* calibration standards as follows:

> Measure the fluorescence of each standard.

> Remove the cuvette from the fluorometer.

> Acidify standard by adding 0.15 mL of 0.1 N HCL (using an autopipet) for every 5 mL of standard solution used.

> *Carefully mix solution* by vortexing at speed "9" for 10 seconds and measure fluorescence of the standard solution again.

Calculate the ratio, r, as follows:

$$r = R_b / R_a$$

Where R_b = Fluorescence of pure chlorophyll-a standard solution before acidification.
R_a = Fluorescence of pure chlorophyll-a standard solution after acidification.

9.0 Procedure

9.1 Sample Preparation

9.1.1 Add 10 mL of 90% buffered acetone (Section 6.2) to the tube containing the filter. Recap tube and invert tube three times making sure that the filter is totally submerged in buffered acetone solution.

9.1.2 Place each tube in an ultrasonic bath, that had been previously filled with water and ice, for 20 minutes.

9.1.3 After 20 minutes, return sample tube to freezer to steep for 16 to 24 hours.

9.2 Sample Analysis

9.2.1 Samples and standards should all be maintained at the same temperature by using a cooler filled with ice.

9.2.2 After the fluorometer has warmed up for at least 15 minutes, use the 90% buffered acetone solution to zero the instrument on the sensitivity setting that will be used for sample analysis.

9.2.3 Following calibration, verify that the flourometer is set at "AUTO RANGE" setting.

9.2.4 Invert sample cuvette four times to mix.

9.2.5 Using a filter flask with a sidearm attached to a vacuum unit, filter entire contents of sample through a GF/F (47 mm) filter, directly into cuvette used for analysis.

Note: Do not let vacuum pressure exceed 1-2 psi or sample volume will be affected

9.2.6 If the concentration of chlorophyll-*a* in the sample is ≥90% of the highest calibration standard, then dilute the sample with the 90% buffered acetone solution and reanalyze.

9.2.7 Record the fluorescence measurement and sensitivity reading used for the sample.

9.2.8 The volume of sample that is to be used for analysis must be known so that correct amount of acid can be added in the pheophytin determination step.

Add 0.15 mL of 0.1 N HCL solution for every 5 mL of extraction solution.

9.2.9 Remove the tube from the fluorometer and acidify the extract using 0.1 N HCL solution.

9.2.10 Mix solution for 10 seconds using a vortex set at speed "9" before measuring fluorescence again.

10.0 Calculations

$$Chlorophyll-a\ (\mu g/L) = (r/r-1)\ (R_b - R_a)$$

$$Pheophytin-a\ (\mu g/L) = (r/r-1)\ (rR_a - R_b)$$

10.1 Determine the chlorophyll-*a* concentration in the sample extract and the pheophytin-*a* concentration in µg/L as follows:

Where R_b *= Fluorescence of sample extract before acidification.*
R_a *= Fluorescence of sample extract after acidification.*
r = The before-after acidification ratio of a pure chlorophyll-a solution (Section 8.2).

10.2 The concentration of chlorophyll-*a* and pheophytin-*a* in the lake water sample is calculated by multiplying the results obtained above by 10 mL (the extraction volume) and dividing this answer by the volume (mL) of the lake water sample that was filtered on the boat. Any other dilution factors should be incorporated accordingly.

11.0 Quality Control

The following audits are to performed:

Audit	Frequency	Limit***
High Check	Once/batch	100 µg ± 15
Low Check	Once/batch	10 µg ± 1.5
Lab Blk.	Once/batch	0.00 µg ± 0.11
Lab Dupl.	Once/batch	RPD 15%
Field Dupl.	Once/batch	RPD 15%
Field Blk.	Once/batch	0.00 µg ± 0.11

*** These limits are estimates based upon data taken from original method and do not pertain to performance data done at CRL. These limits are guidelines. Actual performance limits will still need to be calculated when enough data is available.

12.0 Waste Disposal

Follow all laboratory waste disposal guidelines regarding the disposal of acetone solutions.

13.0 References

13.1 Arar, Elizabeth J. and Collins, Gary B., "In Vitro Determination of Chlorophyll-*a* and Pheophytin-*a* in Marine and Freshwater Phytoplankton by Fluorescence", Environmental Monitoring and Support Laboratory. U.S. EPA 1992.

13.2 Turner Designs Model 10-AU-005 Field Fluorometer User's Manual/November 1992 (P/N 10-AU-075).